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Precise characterisation of component strains is an essential step for placing safe and effective microbial biostimulants on the market

EXECUTIVE SUMMARY

- Characterisation is the first step in the safety evaluation of microorganisms that may be used in agriculture.
- Microbial products can only be characterised precisely by knowing which microbial strains are contained in the product.
- The most accurate way to identify microbial strains is through genomic analysis.
- Taxonomic groupings may allow some general conclusions to be drawn about the strains they contain, but there are limits to using taxonomic groupings as short-hand for the characteristics of the microorganisms in those groups. Taxonomic-based approaches like the Qualified Presumption of Safety (QPS) can simplify data requirements, but rarely eliminate them completely.

I. PROTECTION OF CONSUMERS AND THE ENVIRONMENT STARTS WITH PRODUCT CHARACTERISATION

> *Fundamental Principle:* *Characterisation is the first step in the safety evaluation of microorganisms that may be used in agriculture.*

The number of different micro-organisms used today as plant biostimulant is limited and most of them belong to species whose strains have been demonstrated not to raise safety issues. However, as innovation is driving this industry, it is expected that in the future global portfolio of microbial biostimulants will be very different from the existing portfolio and could potential include strains about which today we have little information.

Innovation in the field of microbiology is based on both the discovery/screening of new species and within a species the selection of more effective, stable and safe strains. (See the annex for a non-exhaustive list of micro-organisms that have been demonstrated to have biostimulant properties.) EU and national authorities need to provide a framework capable of allowing this new, more effective, yet stable and safe strains to market.

EU policy seeks to ensure that consumer and environmental protection are compatible with fostering innovation and economic opportunities. With regard to chemistry-based products, REACH is a critical policy instrument for achieving this objective. There is currently no equivalent to REACH for microorganisms. Instead there are multiple EU frameworks for microorganisms that all have slightly different rules.

The first essential step in the process for companies wishing to place chemicals on the market is to characterise precisely their substance and then conduct hazard and risk assessments. Characterisation of chemical substances takes into account raw materials, product processes and chemical analyses.

Characterisation allows companies to determine to which extent new data must be produced to meet REACH requirements and to which extent publicly available scientific literature can be used or data waivers can be justified. To ensure fair competition, all producer and importers placing substances on the market face same requirements. In practice, the costs related to data production can be shared through data consortia. Furthermore, companies may license the use of data produced by others, if permission is granted and appropriate compensation negotiated.[1] The prices of substances sold to downstream users include a premium to compensate the data producers for their investment.

As the European Union modernises its regulatory framework to promote the bioeconomy, it needs to ensure that bio-based technologies benefit from similar measures to those foreseen for the chemistry-based sectors of the economy. Microbes will need to be characterised in order to determine which data are needed to demonstrate consumer and environmental protection. Regulations need to be able to ensure consumer and environmental protection and yet foster a high level of innovation.

II. CHARACTERISATION OF MICROBIAL PRODUCTS DEPENDS ON IDENTIFYING THE STRAIN(S) THEY CONTAIN

Fundamental Principle: ***Microbial characterisation is dependent on strain identity.***

Whereas the characterisation of chemical products is determined by the substances contained therein, microbial products can only be characterised by knowing which microbial strains are contained in the product.

There are a number of techniques available for accurately identifying microbial strains. Taxonomic names are useful tools for labelling microorganisms, but there are limits to using taxonomic groupings as short-hand for the characteristics of the microorganisms in those groups. (See section IV for more on these limitations.) Identifying the strain is an important first step in understanding to what extent generalisations about the microorganism will be valid at the species or genus level. This is similar to how the characterization of substances based on their chemical identity, raw materials and production processes facilitate choices about the need to test to produce risk assessment data or to waive requirements for specific data.

[1] Data sharing under these conditions is obligatory in cases where it can eliminate the need for animal testing and is optional in other cases.

III. QUALIFIED PRESUMPTION OF SAFETY CAN SIMPLIFY THE SAFETY ASSESSMENT OF MICROORGANISMS

> *Fundamental Principle:* Taxonomy can be a tool to simplify safety assessment, but it is only the first step of the process. The body of knowledge and intended use are important elements to determine to what extent a group of taxonomically related microorganisms can be evaluated together.

According to the European Food Safety Agency's (EFSA) website[2], EFSA's Scientific Committee recommended ion 2007 to adopt a Qualified Presumption of Safety (QPS) approach to its evaluation of safety considerations of biological agents.

The aim of the QPS approach is to harmonise risk assessment and allow risk assessors to focus on the biological agents with the greatest risks or uncertainties.

The QPS approach entails assessing the safety of biological agents by taking into account:

- the definition of the taxonomic unit (establishing identity of the group);
- body of knowledge;
- possible safety concerns (pathogenicity);
- intended end use.

According to EFSA, "If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. **Biological agents included in the QPS list usually undergo a simplified assessment** by EFSA [emphasis added]. In some cases additional specifications ("qualifications") may have to be met which will require a separate assessment. Biological agents not considered suitable for QPS undergo a full safety assessment by EFSA."

In the QPS approach, taxonomy can be a tool to simplify safety assessment, but it is only the first step of the process. The body of knowledge and intended use are important elements to determine to what extent a group of taxonomically related microorganisms can be evaluated together.

The Q in QPS is also important to bear in mind. Presumptions of safety for groups of microorganisms may be subject to specific conditions or qualifications. Thus, relative safety may be presumable for certain contexts and not for others.

[2] http://www.efsa.europa.eu/en/topics/topic/qps

IV. TAXONOMY HAS ITS LIMITS AS A TOOL FOR EVALUATING MICROORGANISMS

The taxonomy of microorganisms is under constant revision

> *Fundamental Principle: The taxonomic system developed for animals and plants is not well-suited for micro-organisms. Micro-organisms are frequently reclassified from one species or genus to another, in part due to our growing understanding about the micro-world.*

The classifications of animals and plants into genera and species – based largely on the capacity of individuals in the same species to reproduce sexually and on visible characteristics-- is relatively well fixed, with reclassifications being relatively rare. This is not the case with microorganisms.

"The vast majority of bacteria are still waiting to be classified [into species] because there's no robust definition of what a bacterial species is,"[3] and some scientists are even beginning to question the usefulness of the concept.[4] As our genetic toolset has evolved rapidly in recent years, previous species groupings have been called into question. One factor is that some microorganisms have been shown to have less shared genetic material than assumed on the basis of their phylogenic characteristics.[5] More problematic for classification, the gene-swapping tendencies of certain strains runs counter to a classification system based on a linear pathway for genetic inheritance.

Microorganisms long-considered to belong to the same species could be (and frequently are) re-classified into two distinct species based on comparison and homology[6]. Because categorisation is not stable, the species and genus labels alone are not sufficient for determining which microorganisms can be presumed to be safe.[7]

Even genera containing well-known species and strains used in agriculture are subject to reclassification:

- Among Trichoderma, most of the strains used as biostimulants or plant protection products have been reclassified into new species. Some *Trichoderma viride* and *Trichoderma harzianum* are now *Trichoderma atroviride* and new species have been described as *Trichoderma gamsii*, just to name a few examples.
- Among *Bacillus subtilis*, some strains are now considered to belong to the *amyloliquefaciens* species or to other more recently described species.

[3] Particularly with regard to human and animal pathogens, phenotypic characteristics such as host range preference and pathogenicity are determinant whereas genotype is used in other classification concepts. The confusion these different classification concepts causes is illustrated by the fact that several genera of well-known pathogens could arguably be seen as being comprised as being part of a single species, but such a reclassification could cause confusion among public health workers with potential consequences for public health.

[4] Hollrichjer, Karin (2007) "Species Don't Really Mean Anything in the Bacterial World". Lab Times. May 2007: pp 22-25.

[5] Borriss R, et al. (2011) "Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7 and FZB42: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons" International Journal of Systematic and Evolutionary Microbiology (2011) 61: 1786–1801; Chen XH et al. (2007) "Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42." Nat. Biotechnol. 25: 1007–1014.

[6] Similarities attributed to a common evolutionary origin.

[7] Indeed a strain could suddenly be included or excluded from a taxonomy-based list due to reclassification regardless of its risk profile. As a consequence, the same strain would be considered acceptable or unacceptable at different times simply because its name had been changed.

Differing pathogenicity, toxicity and antibiotic resistance have been observed among different strains of a single species

> *Fundamental Principle:* ***Functional properties of microbes exhibit a high-level of strain-dependence, in some cases as high as species and genus-level dependence.***

- The scientific literature shows that beneficial strains and pathogenic strains can exist within the same microbial species. Such differences can occur for strains that are very close to each other from a genetic point of view. For example, two Bacillus subtilis species could exhibit totally different effects with respect to the stimulation of plant growth, resistance to antibiotics, and even toxicity.[8]
- Differential effects of micro-organisms in the same group are even more pronounced when considering the genus. As an example, the genus *Pseudomonas* includes some species showing beneficial activities for plants (*Pseudomonas sp*. DSMZ 13134) and some that are plant pathogens (e.g. *Pseudomonas syringae*, *Pseudomonas viridiflava*). Other species, such as *Pseudomonas aeruginosa*, are even considered human pathogens. Yet other strains of *Pseudomonas aeruginosa* are beneficial for plants, like the strain PUPa3. Furthermore, unlike *Escherichia coli*, species of the *Pseudomonas* genus are widely distributed bacteria that can be found in all environmental compartments.
- Antibiotic resistance is one of the characteristics that can vary from one strain to another within a species. The lack of antibiotic resistance is a key criterion for determining whether a microorganism can be used safely in the food chain. For example, EFSA considers that strains of bacteria carrying an acquired resistance to antimicrobial(s) should not be used as animal feed additives, unless it can be demonstrated that the resistance is a result of chromosomal mutation(s).[9]

V. CONCLUSION

Throughout the European Union and the OECD, strain-based approaches are used to evaluation the safety of micro-organisms used in agriculture. The Qualified Presumption of Safety (in the EU) and the Generally Recognized as Safe (in the USA) approaches allow for some generalisations to be made about the safety of certain groups of microorganisms and thus to simplify data requirements for demonstrating the capacity to use these microorganisms safely.

However, the limits to these simplifying frameworks need to be kept in mind when modernising the EU's regulatory framework for placing agricultural microorganisms on the market. Such tools should be used to the extent possible, but they need to be complemented by additional tools for assessing the safety of microorganisms destined for agricultural use at the strain level. Without complementary tools for evaluating microbial safety, the European Union will be placed in the unfortunate position of choosing between protecting consumers and the environment or fostering innovation. (See EBIC Position Paper "How can the European Union Encourage Innovation in Microbial Biostimulants?")

[8] Hoult B and AF Tuxford (1991) "Toxin production by *Bacillus Pumilus*". Journal of Clinical Pathology (1991) 44: 455-458.
[9] "Technical guidance 1: Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance." EFSA Journal (2008) 732: 1-15).

VI. ANNEX – A NON-EXHAUSTIVE LIST OF MICROORGANISM STRAINS, SPECIES AND GENERA FOR WHICH BIOSTIMULANT EFFECTS HAVE BEEN IDENTIFIED[10]

Acetobacter diazotrophicus
Achromobacter piechaudii ARV8
Acinetobacter spp.
Aeromonas spp.
Agrobacterium radiobacter
Alternaria spp.
Azoarcus spp.
Azospirillum brasilense
Azospirillum diazotrophicus
Azospirillum lipoferum
Azotobacter chroococcum
Bacillus atrophaeus
Bacillus edaphicus
Bacillus firmus
Bacillus licheniformis
Bacillus megaterium
Bacillus muciaraglaginous
Bacillus mucilaginous
Bacillus polymyxa
Bacillus pumilus
Bacillus sphaericus
Bacillus spp.
Bacillus subtilis
Bacillus subtilis var. amyloliquefaciens
Beijerinckia spp.
Burkholderia cepacia
Colletotrichum spp.
Comamonas acidovorans
Curvularia spp.
Delftia acidovorans
Erwinia spp.
Flavobacterium spp.
Funneliformis (formerly known as *Glomus*) *mosseae*
Fusarium spp.
Gluconacebacter diazotrophicus
Herbaspirillum seropedicae
Herbaspirillum spp.
Klebsiella pneumoniae
Kluyvera ascorbata
Micrococcus spp.
Neotyphodium spp.
Paenibacillus macerans
Paenibacillus polymyxa
Pantoea agglomerans
Piriformospora indica
Pseudomonas aureofaciens
Pseudomonas chlororaphis
Pseudomonas fluorescens
Pseudomonas mendocina
Pseudomonas putida
Pseudomonas solanacearum
Pseudomonas spp.
Pseudomonas syringae
Serratia entomophila
Staphylococcus kloosii
Streptomyces griseoviridis
Streptomyces spp.
Streptomyces lydicus
Trichoderma spp.
Rhizobia spp.
Rhizophagus irregularis (formerly known as *Glomus intraradices)*
Saccharomyces cerevisae

[10] Sources for this list include: Glick, B.R. (2012) "Review Article: Plant Growth-Promoting Bacteria: Mechanisms and Applications." Scientifica (2012) Article ID 963401:1-15. Available online at http://dx.doi.org/10.6064/2012/963401; du Jardin, P. (2015) "Plant biostimulants: Definition, concept, main categories and regulation." Scientia Horticulturae (2015) 196:3–14; Rouphael, Y. P. Franken et al. (2015) "Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops." Scientia Horticulturae (2015) 196:91-108; Calvo, P., L. Nelson, J.W. Kloepper (2014) "Agricultural uses of plant biostimulants". Plant and Soil (October 2014): 383/1:3-41. Available online at http://rd.springer.com/article/10.1007%2Fs11104-014-2131-8.